REMARKS

This amendment is submitted to cancel claims in order to reduce the filing fee. A cross-reference to related applications has also been added. Claims 11-15, 19 and 23-57 are canceled. There is no new matter added, and entry of the amendment is respectfully requested.

This application contains a Sequence Listing. Applicants enclose a computer-readable form of the Sequence Listing. The content of the paper copy of the Sequence Listing and of the computer readable form is the same.

The Examiner is hereby invited to contact the undersigned by telephone if there are any questions concerning this amendment or application.

Respectfully submitted,

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For: Pullulanase Variants and Methods for Preparing Such Variants With Predetermined Properties

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Sir:

Below is a marked-up version of the amendments made in the accompanying amendment.

IN THE CLAIMS:

The claims have been amended as follows:

1. (Unchanged.) A method for producing a variant of a parent pullulanase, the variant having at least one altered property as compared to the parent pullulanase, the method comprising:

a) modeling the parent pullulanase on the three-dimensional structure of SEQ ID NO: 1 depicted in the Appendix to produce a three-dimensional structure of the parent pullulanase;

b) identifying in the three-dimensional structure obtained in step (a) at least one structural part of the parent pullulanase, wherein an alteration in said structural part is predicted to result in an altered property;

c) modifying the nucleic acid sequence encoding the parent pullulanase to produce a nucleic acid sequence encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and

d) expressing the modified nucleic acid sequence in a host cell to produce the variant pullulanase.

2. (Unchanged.) The method according to claim 1, wherein the altered property is pH dependent activity, thermostability, substrate cleavage pattern, specific activity of cleavage, substrate specificity, such as higher isoamylase activity and/or substrate binding.

3. (Unchanged.) The method according to claim 2, wherein the altered property is a higher isoamylase activity as defined by an increase of at least 5% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10% w/v rabbit liver glycogen solution for a period of 10 min.

4. (Amended.) The method according to claim[s] 1 [or 2], wherein the altered property is an improved thermostability as defined by differential scanning calorimetry (DSC) using the method described herein.

5. (Amended.) The method according to claim[s] 1 [or 2], wherein the altered property is an improved thermostability as defined by an increased half-life ($T_{½}$) of at least about 5%[, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%,] in the "$T_{½}$ assay for liquefaction" described herein, using a pH of 5.0 and a temperature of 95ϴC.

6. (Amended.) The method according to claim[s] 1 [or 2], wherein the altered property is an improved thermostability as defined by an increased residual enzyme activity of at least about 5%[, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%,] in the "assay for residual activity after liquefaction" described herein, using a pH of 5.0 and a temperature of 95ϴC.

7. (Amended.) The method according to claim[s] 1 [or 2], wherein the altered property is an improved thermostability as defined by an increased half-life ($T_{½}$) of at least about 5%[, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%,] in the "$T_{½}$ assay for saccharification" described herein, using a pH of 4.5 and a temperature of 70ϴC.

8. (Amended.) The method according to claim[s] 1 [or 2], wherein the altered property is an improved thermostability as defined by an increased residual enzyme activity of at least about 5%[, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%,] in the "assay for residual activity after saccharification" described herein, using a pH of 4.5 and a temperature of 63ϴC.

9. (Unchanged.) The method according to claim 8, wherein the "assay for activity for saccharification" described herein, is carried out at a pH of 4.5 and at a temperature of 70ºC.

10. (Unchanged.) A method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying an internal or external cavity or crevice in the three-dimensional structure of the parent pullulanase;

b) substituting at least one amino acid residue in the neighborhood of the cavity or crevice with another amino acid residue which increases the hydrophobic interaction and/or fills out or reduces the size of the cavity or crevice;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the thermostability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased thermostability as compared to the parent pullulanase.

16. (Unchanged.) A method for constructing a variant of a parent pullulanase, where the variant pullulanse has an altered substrate specificity as compared to the parent pullulanase, the method comprising:

a) identifying the substrate binding area in a model of the three-dimensional structure of the parent pullulanase;

b) modifying the substrate binding area by an amino acid substitution, deletion and/or insertion;

c) optionally repeating step b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps a) - d);

f) testing the substrate specificity of the variant;

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having an altered substrate specificity as compared to the parent pullulanase.

17. (Unchanged.) The method according to claim 16, wherein the altered substrate specificity is an increased isoamylase activity compared to the parent pullulanase.

18. (Unchanged.) The method according to claim 17, wherein the increased isoamylase activity is defined by an increase of at least 5% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10% w/v rabbit liver glycogen solution for a period of 10 min.

20. (Amended.) A method according to [any of the preceding claims] claims 1, 10 or 16, wherein the parent pullulanase has more than 40% homology with the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5[, preferably more than 50%, such as more than 60%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% homology with the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.]

21. (Unchanged.) A method according to claim 20, wherein the parent pullulanase has the amino acid sequences shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

22. (Amended.) A method for producing a pullulanase variant, the method comprising:

a) constructing the variant by the method according to [any of claims 10-21] claim 10;

b) transforming a microorganism with a DNA sequence encoding the variant;

c) cultivating the transformed microorganism under conditions which are conducive for producing the variant; and

d) optionally, recovering the variant from the resulting culture broth.